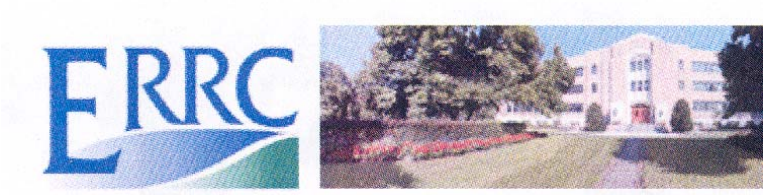

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Title: Agarose/Agar Assay System for the Selection of Bacteriocin-Producing Lactic Fermentation Bacteria

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Agarose/agar assay system for the selection of bacteriocin-producing lactic fermentation bacteria

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Abstract

The direct selection of bacteriocin-producing lactic fermentation bacteria was possible by plating diluted cultures of *Pediococcus acidilactici* on mixed agarose agar layers with the amount of each component incrementally adjusted to 1.2% (w/v). Between 0.5 and 1% agarose, the increased flexibility of the solidified support layer allowed its removal from Petri dishes without tearing and its smooth layering on the surface of 1.5% (w/v) standard agar medium seeded with *Listeria innocua* as the test organism. Selection of bacteriocin-producing clones was based on the size of inhibition zones visible in the bottom agar layer under colonies growing on the agarose/agar top layer. The lack of contact with the test organism permitted the transfer of superior clones from the surface of the agarose/agar layer directly into an appropriate nutrient medium.

Introduction

Bacteriocins are chromosomally or plasmid-encoded, usually narrow-range antimicrobial peptides with bactericidal activity. Many strains of lactic fermentation bacteria (LFB), some with important applications in the production of fermented dairy and meat foods, produce bacteriocins (Jack *et al.* 1995). Certain bacteriocins of LFB (lactobacilli, lactococci, leuconostocs, micrococci, pediococci, and streptococci) are active against food-borne pathogens including *Bacillus cereus, Clostridium perfringens, Staphylococcus aureus* and *Listeria* species.

Identification of new and superior bacteriocin-producing strains of LFB is important since there is a growing interest in the selective application of bacteriocins with broader antimicrobial spectrum (nisin, pediocin, lacticin) as natural bioprotective agents in food systems to reduce the incidence of bacterial food poisoning caused by *Listeria* and other pathogens (Holzapfel *et al.* 1995, Stiles 1996, Ross *et al.* 1999, Castellano *et al.* 2001). Nisin, the product of some strains of *Lactococcus lactis* subsp. *lactis*, which was accorded GRAS (generally recognized as safe) status and approved for food use by the U.S. Food and Drug Administration (Federal Register 1988), has already found a variety of applications in food preservation (Molitor & Sahl 1991). Since toxicity to humans has not been linked to any known bacteriocin produced by LFB, future FDA approval of other bacteriocins (e.g. pediocin, lactacin) is anticipated for food and possibly biomedical uses. Therefore, research has remained intensive on developing optimum conditions for the production of selected bacteriocins with high potential for applications in food processing (Morgan *et al.* 1999, Guerra & Pastrana 2001).

Most methods for identifying bacteriocin- producing cultures have been derived from those producing antibiotics and are based on the diffusion of the antimicrobial peptides in solid or semisolid agar media leading to the inhibition of a sensitive target organism (Parente *et al.* 1995). In *direct* assay techniques, wells cut in the agar layer and seeded with the test organism are filled with culture broth samples (Tagg *et al.* 1976). Alternatively, a defined amount (5–10 μl) of each undiluted or diluted sample may also be spotted

directly on the agar surface, followed by incubation to detect zones of inhibition. In variations of the *indirect (deferred)* assay techniques, samples of cultures are spotted or streaked on agar surfaces. After colonies develop, usually within 24 to 48 h, the test plate is overlaid with an agar film seeded with the target organism and incubation is continued for 24 h or until growth is evident and inhibition zones are detectable. However, the recovery of isolated bacteriocin-producing clones from overlaid agar plates is cumbersome and frequently requires the elimination of contaminating test microbe used in the screening assay. Other indirect agar diffusion assays for detecting antimicrobial activity use both sides of the agar disc to avoid contact between producing and test organisms and include the flip-streak method in which the agar discs are inverted (Kekessy & Piguet 1970) and the double-sided plate method that requires special culture dishes (Colwell & Speidel 1985).

In this report we describe a double-layer bioassay technique for the direct selection of superior bacteriocin-producing strains. The method uses mixed agarose/agar top layers to support the surface growth of *Pediococcus acidilactici* and to prevent contact with the Listeria test organism inoculated into the bottom standard agar layer. The technique permits the screening of a large number of colonies of pediococci or other types of LFB for bacteriocin activity.

Materials and methods

Microbial strains and culture conditions

Cultures used were maintained as frozen stocks at −70 °C and before experimental use were transferred twice in broth for 16 h. The pediocin-producing *Pediococcus acidilactici* strain F culture with the bacteriocin gene on a 7.8 kb plasmid (Ray *et al.* 1989) was maintained in MRS medium (Difco Laboratories, Detroit, MI) at 37 °C. *Listeria innocua*, the test organism for detecting pediocin activity, was grown in brain heart infusion (BHI, Difco) medium at 30 °C. The amount of bacteriocin produced by *P. acidilactici* strain F after 24 h was estimated by a serial twofold dilution assay (Pilet *et al.* 1995) as described previously (Coderre & Somkuti 1999). The spotting of 5 μl samples of cell-free medium on *L. innocua* indicator plates was followed by holding for 6 h at 6 °C before incubation for 16 h at 30 °C.

Agarose/agar assay procedure

Solid medium for propagating the test microbe *L. innocua* was prepared by the addition of bacteriological agar (Difco) to BHI broth at 1.5% (w/v). The medium was inoculated with 1% (v/v) of a 16-h-old broth culture of *L. innocua* before pouring ca. 3 mm deep layer in 24.5 × 24.5 cm Nalge Nunc bioassay plates (Nalge Nunc Intnl., Naperville, IL). After solidifying, the plates which constituted the bottom layers in the experimental assay procedure were stored at 6 °C before use.

Solid MRS media for growing *P. acidilactici* strain F were prepared by using mixtures of gel electrophoresis grade Seakem ME agarose (FMC BioProducts, Rockland, ME) and bacteriological agar in which the combined concentration of the two components was 1.2% (w/v). The concentration of individual components was incrementally adjusted from 0 to 1.2%. The molten medium was poured into standard 9 cm Petri plates to form a ca. 2.5–3 mm deep layer. Solidified plates were inoculated by spreading 25–100 μl samples of a serially diluted 16 h MRS broth culture of *P. acidilactici* strain F and incubated for 16–24 h at 37 °C.

To identify and select bacteriocin-producing *P. lactici* colonies, the agarose/agar discs of plates with 25–100 colony forming units (c.f.u.) of pediococci were removed with a sterile wooden-handle spatula with a 16.5 × 100 mm stainless steel blade, and layered on the BHI Listeria agar plates. The procedure was carried out under aseptic conditions in a laminar-flow biosafety cabinet. The assembled assay plates were held at 6 °C for varying lengths of time before transfer to a 30 °C incubator for up to 16 h. The plates were scored for the presence of inhibition zones resulting from the diffusion of antimicrobial substances produced by *P. acidilactici* colonies on the top layer.

Results

Agarose/agar combinations and bacterial growth

Manipulation of standard agar plates is difficult because of the susceptibility of the solidified medium to splitting and tearing. To circumvent this problem, we attempted to increase the resilience and flexibility of the support layer by testing several combinations of gel electrophoresis grade agarose and standard bacteriological agar as solidifying agents in MRS medium. The total concentration of the two solidifying agents

was kept constant at 1.2% (w/v) while the concentration of each component was adjusted stepwise between 0 and 1.2%. With each type of medium, the removal of the solidified agarose/agar layer was attempted with a sterile spatula. We found that by incrementally increasing the agarose concentration from 0% to 1% in the mixture, the solidified MRS support layer became increasingly more flexible and resistant to tearing during manipulations. Further experiments confirmed that between 0.6 and 1% agarose concentration, the incidence of tearing was negligible when the 2.5–3 mm thick 85 mm diameter discs of solid MRS agarose/agar support medium were scored and lifted out of Petri dishes with a sterile spatula fitted with a flexible blade, and carefully layered on regular BHI Listeria agar (1.5%) poured in rectangular bioassay plates (Figure 1).

The size and appearance of *P. acidilactici* colonies grown for 16–24 h on mixed support media were also evaluated. In MRS support media prepared with a mixture of 80% agarose and 20% agar, surface colonies of pediococci were normal in appearance and could not be distinguished from colonies grown on standard 1.2% MRS agar.

Assay procedure

The laminar flow biological safety cabinet provided a convenient and protected environment for carrying out the entire assay procedure. Based on the results of multiple trials using various combinations of agarose/agar mixtures to obtain a flexible and manageable support layer, the MRS medium was routinely solidified with a mixture of 0.7% agarose and 0.5% agar for the preparation of *P. acidilactici* spread plates. After 16 h incubation at 37 °C, MRS agarose/agar discs were lifted out of Petri dishes with a sterile spatula and layered on top of the BHI agar inoculated with *Listeria* (Figures 1a–d). Because of its increased flexibility, the top layer could be lifted up and repositioned as needed to prevent wrinkling and to eliminate large air bubbles trapped between the two layers. Alternatively, if the plate was not overcrowded with colonies, larger pockets of air could be dislodged by applying gentle pressure with the sterile spatula. Air bubbles trapped under the MRS top layer with less than 2 mm in diameter did not interfere with the development of inhibition zones and the evaluation of the assay plates. However, agarose/agar discs less than 2.5 mm in depth is not recommended since they were more prone to wrinkling when picked up with the spatula, making it more difficult to deposit them smoothly on the test agar layer.

The bioassay plates, each overlaid with 4–5 MRS agarose/agar discs (Figure 1), were incubated at 30 °C to allow for the growth of *L. innocua*. Assay plates were usually scored for inhibition zones after 16 h of incubation. However, if incubation was carried out at 37 °C, zones of inhibition were already discernible after 4 h.

Effect of incubation time on Pediococcus *growth*

The efficiency of the bioassay procedure and the interpretation of the results were influenced by several variable conditions (Figures 2a–d). Longer incubation increased the size of *Pediococcus* colonies, which resulted in proportionately larger inhibition zones in the BHI Listeria agar layer (Figures 2a and 2b). In extreme cases, when *Pediococcus* colonies exceeded 2 mm in size following incubation at 37 °C for 24 to 48 h, confluent inhibition zones developed in the BHI Listeria agar layer (Figure 2c), which interfered with the identification of bacteriocin-negative colonies. However, confluent zones were less significant in cases where only a few bacteriocin-producing colonies were present on relatively crowded MRS agarose/agar plates (Figure 2d). The results of multiple experiments indicated that the preferred *Pediococcus* colony diameter is ca. 0.5 mm (or less) to yield compact inhibition zones in the *Listeria* test plates. This requirement may be met by incubating the *Pediococcus* MRS agarose/agar plates at 37 °C for not longer than 16 h, which permits the screening of more colonies per plate to identify non-producing and superior bacteriocin-producing strains (Figure 2a).

Effect of preincubation contact on inhibition zones

The duration of preincubation contact between *Pediococcus* and *Listeria* plates also influenced the outcome of bioassays. As the length of preincubation contact at 6 °C between the top *Pediococcus* MRS agarose/agar discs and the bottom *Listeria* BHI agar plates increased, larger inhibition zones developed in the bottom layer during incubation at 30 °C, due to the diffusion of larger amounts of bacteriocin. If the contact time was longer than 2 h, inhibition zones frequently became confluent, resembling in appearance the plates shown in Figures 2b and 2c. Repeated experiments determined that preincubation contact between the 16 h *Pediococcus* colonies on the top MRS agarose-agar and the BHI Listeria agar at 4–6 °C may

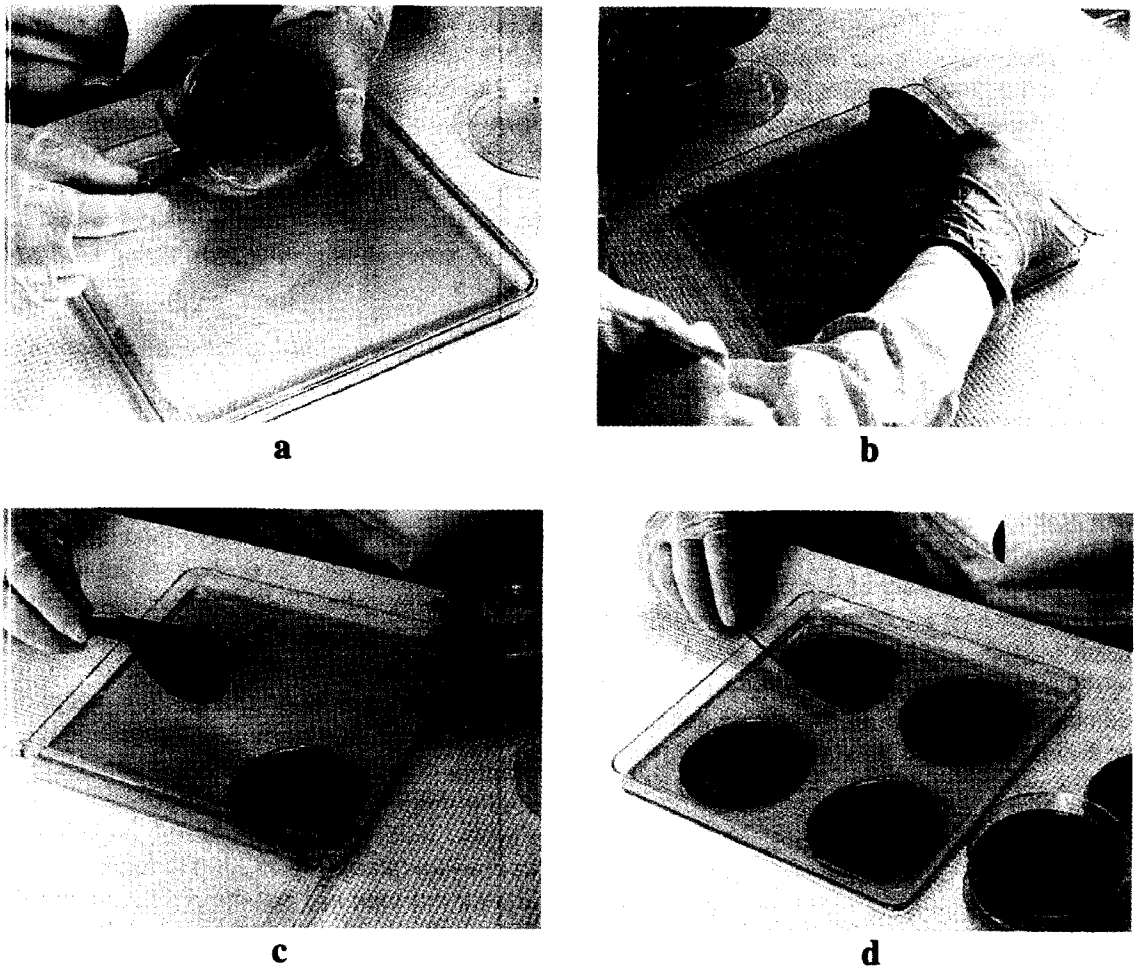

Fig. 1. Preparation of double-layer bioassay plates. Top layer: MRS agarose/agar discs with 16 h colonies of *Pediococcus acidilactici*. Bottom layer: BHI Listeria agar. (a)–(d) Deposition of flexible top agarose/agar layers with a sterile spatula.

be reduced to 30 min or less to achieve compact zones of inhibition similar to that shown in Figure 2a.

Discussion

Most techniques for detecting bacteriocin activity are based on the diffusion of the antimicrobial peptide through an agar medium. The original indirect ('flip-streak') method of Kekessy & Piguet (1970) which involved using both sides of an agar plate for growing the bacteriocin-producing and the target organism has been used with minor adaptations (Barefoot & Klaenhammer 1983, Lewus & Montville 1991, Gupta & Batish 1992). Other modifications reported generally require the overlaying of streaks or single colony isolates of putative bacteriocin producers with a lawn of indicator microorganism (Bhunia *et al.* 1988, Pucci *et al.* 1988, Harding & Shaw 1990, Joerger & Klaenhammer 1990, Coderre & Somkuti 1999, Oscariz *et al.* 1999). Although suitable for bacteriocin detection, all variations of the indirect assay method involving an overlay step have the drawback of having direct contact between the bacteriocin-producing colonies and the test organism. Thus, the isolation of bacteriocin-producing clones as pure cultures is difficult and frequently requires time-consuming, multistep manipulations. In addition, maneuvers performed in the flip-streak method and all variations of the indirect overlay assay frequently cause the smearing and distortion of streaks or colonies of producing cultures.

The double-layer bioassay method described in this study avoids the shortcomings of the deferred (indirect) overlay assays by preventing contact between producing colonies and the target organism. Unlike the double-sided plate assay which also pre-

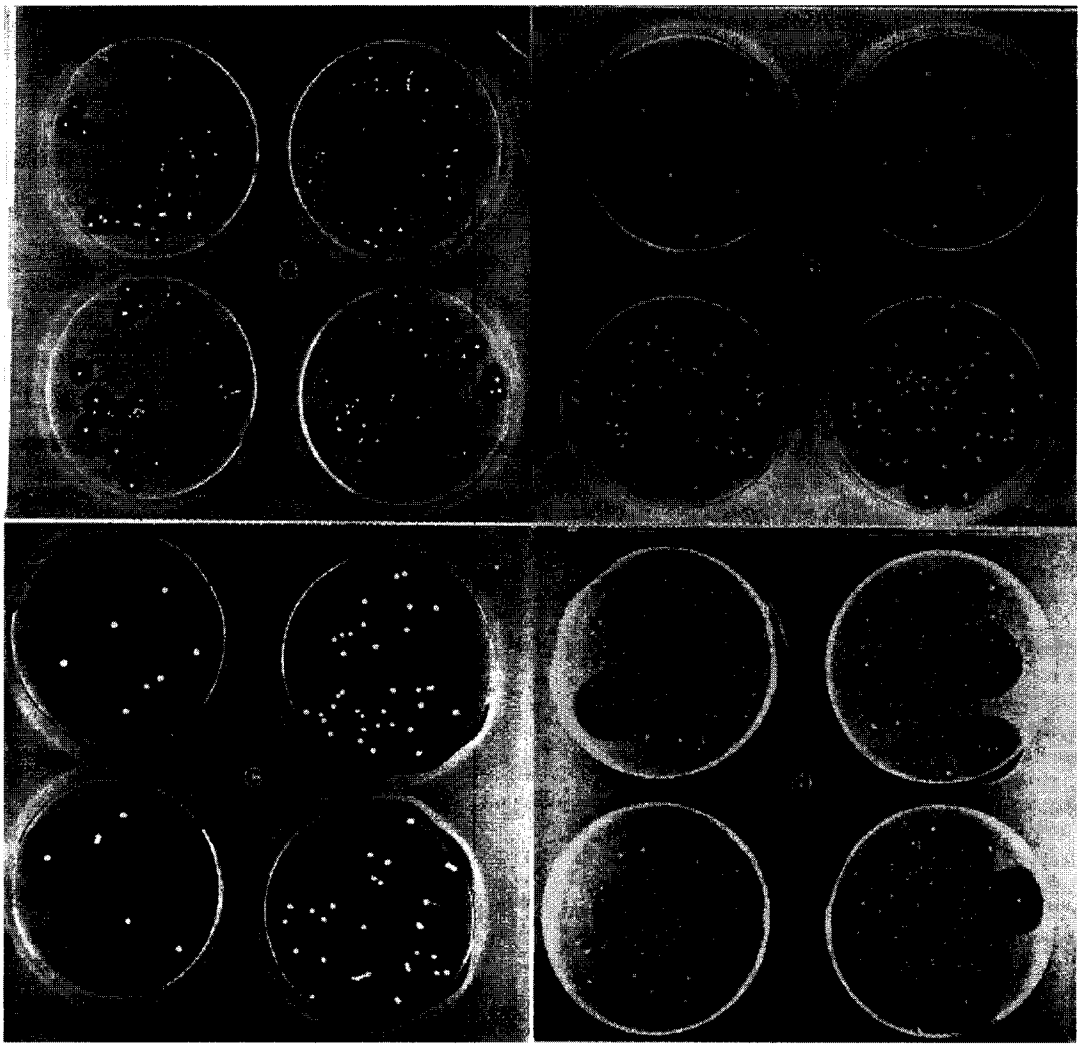

Fig. 2. Effect of preincubation conditions on the detection of bacteriocin-producing colonies. (a) Compact inhibition zones obtained with 16 h *Pediococcus acidilactici* culture (colony diam.: ca. 0.5 mm) following preincubation contact with *Listeria* test medium for 30 min or less. (b) and (c) Increased diameter inhibition zones obtained with 24–40 h *Pediococcus colonies*; similar phenomena were detected after extending preincubation contact between the layers from 30 min to 4 h and 16 h, respectively. (d) Mixture of bacteriocin-producing and nonproducing colonies.

vents cross contamination (Brown 1982), it does not require custom-made culture dishes. The technique relies on using mixtures of agarose (0.5% or higher) and standard agar (0.7% or lower) as solidifying agents at 1.2% (w/v) total concentration, which imparts greater flexibility to the support medium and allows its manipulation. Although the growth characteristics of pediococci remained unaltered between 0.5 and 1% (w/v) agarose, a mixture of 0.7% agarose and 0.5% standard agar was routinely used as the solidifying agent in the preparation of top layers. For the development of visible inhibition zones, preincubation contact between the two layers could be entirely eliminated or kept under 30 min. If incubation of the bioassay plates was carried out at 37 °C instead of 30 °C, inhibition zones already became discernible after 4 h. In our model system, the colonies of bacteriocin-producing pediococci on the surface of the top agarose/agar layer corresponding to clear zones in the lower Listeria test medium could be picked directly into an appropriate production medium, without concern for possible contamination by the Listeria test organism. Following the isolation of selected bacteriocin-producing clones, culture productivity under different conditions may be assessed by rapid detection methods based on mea-

suring K^+ efflux from a bacteriocin sensitive strain (Mugochi *et al.* 2001),

The double-layer agarose/agar assay method reported here is suitable for screening a large number of colonies from natural sources or identifying new bacteriocin-producing strains in mixed microbial populations commonly present in food systems processed with LFB. It is also a convenient technique for recapturing superior bacteriocin-producing clones of strains in which plasmid borne bacteriocin production may be subject to loss by plasmid curing or segregational instability.

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